



Research Paper

Long noncoding RNA UCA1 promotes osteosarcoma metastasis through CREB1-mediated epithelial-mesenchymal transition and activating PI3K/AKT/mTOR pathway



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ABSTRACT

Increasing evidences have demonstrated that Long noncoding RNAs (lncRNAs) are key regulatory RNAs that participate in multiple biological processes. LncRNA urothelial carcinoma-associated 1 (*UCA1*) is a newly identified lncRNA and functions as a regulator of growth in several cancers. However, the biological function and molecular mechanism of *UCA1* in the metastasis of osteosarcoma remain unclear. In this study, we firstly found *UCA1* is upregulated in both osteosarcoma tissues and cell lines, and increased *UCA1* is associated with higher tumor stage, larger tumor size and poorer prognosis. Then for the first time, we demonstrated that *UCA1* promotes the invasion and metastasis of osteosarcoma both *in vitro* and *in vivo*. Further mechanistic investigation showed that *UCA1* directly interacts with miR-582 and suppresses its expression. Moreover, *UCA1* increases CREB1 expression by functioning as a ceRNA against miR-582, thus promoting the EMT process via CREB1-mediated PI3K/AKT/mTOR pathway and finally leading to osteosarcoma metastasis. These findings may extend the function of *UCA1* in osteosarcoma progression and provide a promising therapeutic target for osteosarcoma treatment.

1. Introduction

Osteosarcoma is the most frequent primary bone tumor which mainly occurs in adolescents and children [1]. In the past few decades, despite advancements in complete surgical resection and neoadjuvant chemotherapy for osteosarcoma patients, the prognosis is still unfavorable due to the high rate of metastasis. The 5-year survival rate of patients with osteosarcoma having distant metastases is approximately 30% [2]. Therefore, understanding of the molecular mechanisms of osteosarcoma metastasis is important to improve the prognosis of osteosarcoma patients.

Long noncoding RNAs (lncRNAs) are typically longer than 200 nucleotides with little or no protein coding capacity. Accumulating evidences indicate that lncRNAs are aberrantly expressed in many human tumors and play critically important roles in diverse biological processes, such as proliferation, apoptosis and metastasis [3]. LncRNAs regulate gene expressions through multiple mechanisms, including modulating transcription, indirect degradation of RNA transcripts and regulating various post-transcriptional processes [4].

Urothelial carcinoma-associated 1 (*UCA1*), which is a novel identified lncRNA, is widely expressed in various tumors and plays key roles in multiple biological processes [5]. Previous studies have demonstrated that *UCA1* was highly expressed in ovarian cancer [6], non-small cell lung cancer [7] and prostate cancer [8]. *UCA1* was initially found in bladder cancer and could enhance the tumorigenicity of cancer cells both *in vitro* and *in vivo* [9]. Bioinformatic analyses showed that *UCA1* was highly expressed in tumor tissues from 361 gastric cancer patients and acted as an oncogene to promote cell proliferation. *UCA1* also functions as a tumor promoter in colorectal cancer [10]. It was reported that high expression of *UCA1* was associated with tumor proliferation and indicated worse prognosis in colorectal cancer [5]. In osteosarcoma, previous study has found that *UCA1* was overexpressed in osteosarcoma and promoted cell growth. However, there has been no research focusing on the role of *UCA1* in osteosarcoma metastasis. Therefore, to explore how *UCA1* participates in osteosarcoma progression, especially its effect on the invasion and migration of osteosarcoma cells, aiming at discovering novel targets for treatment becomes our goal of study.

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In this study, we validated that the *UCA1* was significantly upregulated in osteosarcoma and, for the first time, we confirmed that *UCA1* promotes the metastasis of osteosarcoma both *in vitro* and *in vivo*. Also, we are the first to demonstrate that *UCA1* interacts with and suppresses miR-582 as a potential ceRNA. The pro-metastatic effect of *UCA1* is achieved by inhibiting the epithelial-mesenchymal transition (EMT) via CREB1-mediated PI3K/AKT/mTOR pathway.

2. Materials and methods

2.1. Cell culture and tissue samples

Normal osteoblast cell line hFOB1.19, osteosarcoma cell lines Saos-2, HOS, U2-OS and MG-63 were obtained from the Cell Bank of Chinese Academy Sciences. The hFOB1.19 cells were cultured in α -MEM/F-12, Saos-2 in McCoy's 5A, U2-OS cells in RPMI-1640 and HOS cells in MEM. All of the media and the supplemented 10% fetal bovine serum were from Gibco. All cells were incubated at 37 °C in a 5% CO₂ atmosphere.

Human tissue samples of paired normal and primary tumor tissues from 30 osteosarcoma patients were collected from No. 215 Hospital of Shannxi Nuclear Industry. The study was approved by the Medical Ethics Committee and written informed consents were obtained from all patients.

2.2. RNA extraction and quantitative real-time PCR (qPCR)

Total RNA from both tissue samples and cell lines were extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT Reagent Kit (TaKaRa) for detecting mRNA, lncRNA and miRNA levels. Real-time PCR was performed in triplicate using SYBR Premix Ex Taq (TaKaRa) on CFX96 Real-Time PCR Detection System (BioRad). Expression levels of mRNAs, lncRNAs and miRNAs were normalized to GAPDH, 18S rRNA and U6, respectively.

2.3. Oligonucleotides, plasmids construction and cell transfection

The Control/*UCA1* shRNA and the full-length human *UCA1* sequence were synthesized by Genscript (Nanjing, China) and then subcloned into pLKO.1 and pCDNA3.1 vectors, respectively. According to the manufacturer's instructions, cells were transfected with Control/*UCA1* shRNA vectors or Control/*UCA1* vectors using Lipofectamine[®] 2000 reagent (Invitrogen) at final concentrations of 2 μ g/ml for 48 h.

For stable overexpression of *UCA1*, the synthesized full-length *UCA1* cDNA was cloned into lentiviral expressing vector pLV-puro. After production of lentiviral particles according to the standard protocols, cells were transfected for 24 h with 2 μ g/ml polybrene (Sigma) and selected by using 2 μ g/ml puromycin for 7 days.

The synthetic miR-NC/miR-582 and inhibitor NC/miR-582 inhibitor purchased from GenePharma (Shanghai, China). All of the oligonucleotides were transfected into cells using Lipofectamine[®] 2000 reagent (Invitrogen) at final concentrations of 50 nM.

2.4. CCK8 assay and apoptosis assay

Cell proliferation was detected by a Cell Counting Kit-8 (CCK-8) assay (Dojindo) as manufacturer's instructions. Cells were seeded into 96-well plates (2 \times 10³ cells/well) for 1, 2, 3 and 4 days' observation. Cell apoptosis was detected by using Apoptosis Detection Kit (KeyGEN) according to the manufacturer's instructions. Cells were stained with fluorescein isothiocyanate-conjugated Annexin V and PI and analyzed with a FACScan flow cytomete.

2.5. Wound-healing assay and matrigel invasion assay

For the wound-healing assays, wound closures were observed by

taking photographs under a microscope 0 and 48 h after scratching. Matrigel invasion assays were performed with Matrigel (BD) and 8 μ m, 24-well trans-well chambers (Millipore) following the manufacturer's instructions. Cells (1 \times 10⁵) in 200 μ l of serum-free medium were added to the upper chamber and cultured for 48 h. Migrated cells were fixed with ethanol and stained with 0.1% crystal violet and photographs were taken of 6 randomly selected fields for each well.

2.6. Western blot analysis

Western blotting was performed according to the standard protocol. In brief, cell lysates quantified with the BCA method were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated with 1:1000 primary antibodies and then 1:5000 goat anti-mouse or goat anti-rabbit IgG-HRP conjugate secondary antibodies (Akea). Blots were detected by ECL system (Alpha Innotech) according to the manufacturer's instructions.

2.7. Animal models

To evaluate the lung metastatic potential of osteosarcoma cells *in vivo*, 5 \times 10⁶ U2-OS/Lenti-Control or U2-OS/Lenti-*UCA1* cells in 200 μ l of serum-free medium were injected into six-week-old, male nude mice through the tail vein (n = 6 per group). Four weeks later, the mice were sacrificed. Individual organs from the mice were removed, and metastatic tissues were analyzed with H&E staining.

2.8. Luciferase reporter assay

The *UCA1* WT/MT 3'-UTR and CREB1 WT/MT 3'-UTR sequences were cloned into pmirGLO plasmid, respectively. The vectors (2 μ g/ml) were co-transfected with miR-NC/miR-582 or inhibitor NC/miR-582 inhibitor (50 nM) into cells by Lipofectamine[®] 2000 reagent (Invitrogen). Luciferase activity was determined by the Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.9. RNA immunoprecipitation (RIP) assay

Cells were co-transfected with pLV-MS2 or pLV*UCA1*-WT-MS2 or pLV-*UCA1*-MT-MS2 and pMS2-GFP (Addgene). After 48 h of transfection, cells were subjected to a RIP assay by using 5 μ g GFP antibody (Sigma-Aldrich) or negative control IgG using RNA Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. For anti-AGO2 RIP, cells were transfected with miR-NC/miR-582. After 48 h of transfection, cells were used to perform anti-AGO2 RIP assay using 5 μ g anti-AGO2 antibody (Millipore) as described above.

2.10. Statistics analysis

Data were analyzed using the SPSS 19.0 software and expressed as the mean \pm standard error of the mean (s.e.m) from at least three separate experiments. Two tailed Student's t-tests were used to evaluate statistical significance between two independent groups of samples. The significance of correlations between miR-582 and *UCA1* levels were judged via the Pearson product-moment correlation coefficient. Differences were considered significant when * p < 0.05 and *** p < 0.001.

3. Results

3.1. *UCA1* is upregulated in osteosarcoma and promotes its growth

To detect the expression level of *UCA1* in osteosarcoma, strictly

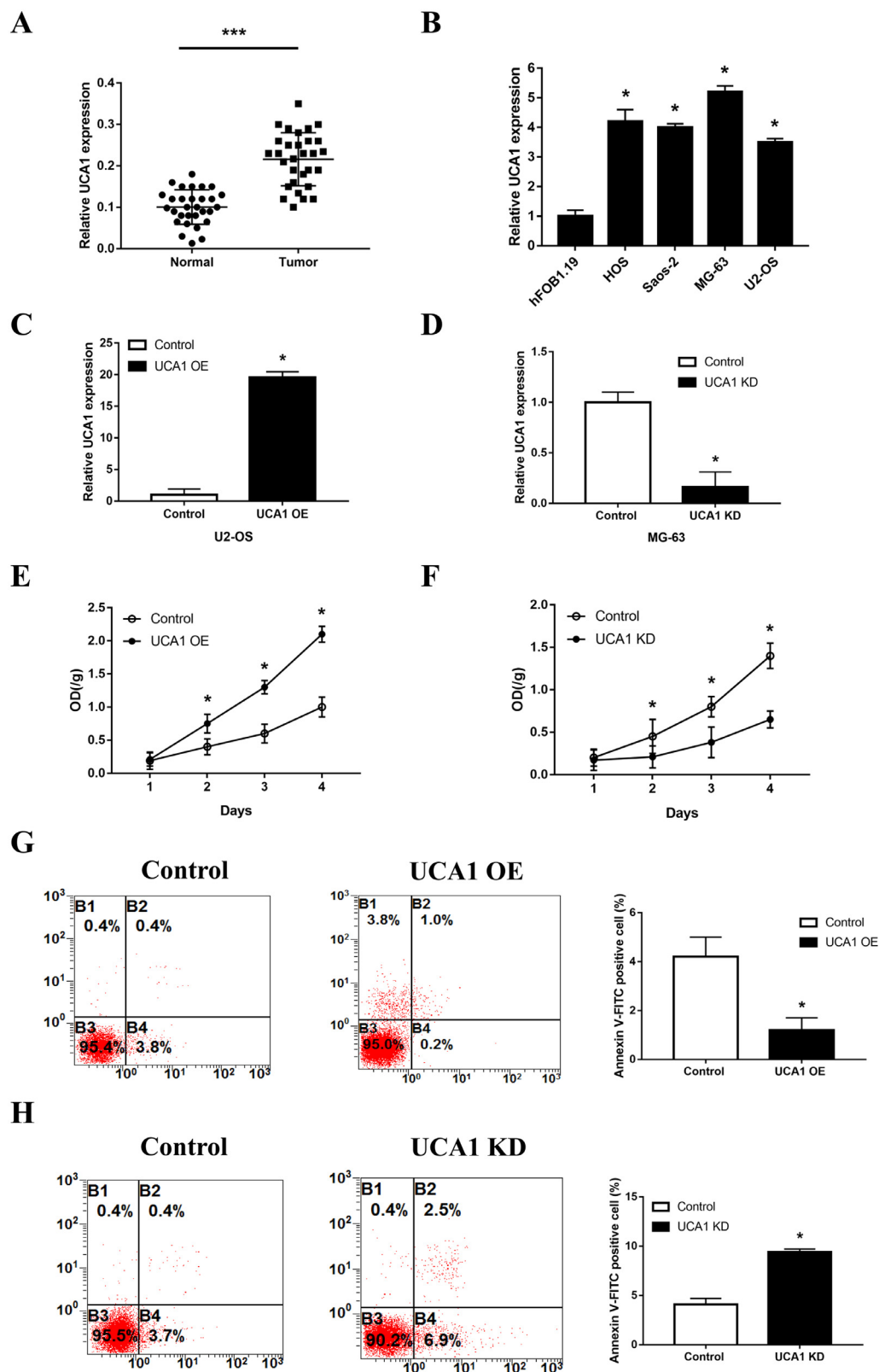


Fig. 1. UCA1 is upregulated in osteosarcoma and promotes its growth. (A) Expression levels of *UCA1* in paired normal and tumor tissues from 30 osteosarcoma patients detected by qRT-PCT ($p < 0.0001$). (B-D) Expression levels of *UCA1* in osteosarcoma cell lines (B, HOS vs hFOB1.19, $p = 0.0002$; Saos-2 vs hFOB1.19, $p < 0.0001$; MG-63 vs hFOB1.19, $p < 0.0001$; U2-OS vs hFOB1.19, $p < 0.0001$), U2-OS cells transfected with pHLV Control/*UCA1* vectors (C, $p < 0.0001$) and MG-63 cells transfected with Control/*UCA1* shRNA (D, $p = 0.0013$) detected by qRT-PCT. (E-H) CCK8 assays (E, F) and apoptosis assays (G, H) carried out in U2-OS cells transfected with pHLV Control/*UCA1* vectors (E, *UCA1* overexpression vs control: day2 $p = 0.0303$, day3 $p = 0.0021$; day4 $p = 0.0006$; G, $p = 0.0053$) and MG-63 cells transfected with Control/*UCA1* shRNA (F, *UCA1* knockdown vs control: day2 $p = 0.0463$, day3 $p = 0.0282$; day4 $p = 0.002$; H, $p = 0.0002$). * $P < 0.05$ and *** $P < 0.001$ (Student's *t*-test).

paired primary tumor tissues and normal tissues were collected from 30 osteosarcoma patients. As displayed in qPCR assays, *UCA1* expression was significantly upregulated in osteosarcoma tissues, with the average level over two folds higher than that in normal tissues (Fig. 1A). Similarly, compared with the normal osteoblast cell line hFOB1.19, osteosarcoma cell lines Saos-2, HOS, U2-OS and MG-63 all showed

increased level of *UCA1* (Fig. 1B). The following clinical analysis showed that increased *UCA1* expression was significantly associated with metastasis, larger tumor size and higher clinical stage of osteosarcoma (Table 1). Given the upregulated *UCA1* in both the tumor tissues and cell lines, we deduced that *UCA1* may promote the growth of osteosarcoma. To clarify this, U2-OS cells, which had the lowest level

Table 1
The relationship between UCA1 expression and clinicopathological features in osteosarcoma patients.

| | UCA1 expression levels | | P |
|-----------------|------------------------|------|-------|
| | Low | High | |
| Age (years) | | | |
| ≤ 18 | 8 | 9 | 0.713 |
| > 18 | 7 | 6 | |
| Gender | | | |
| Female | 9 | 10 | 0.705 |
| Male | 6 | 5 | |
| Location | | | |
| Femur/ Tibia | 11 | 10 | 0.69 |
| Elsewhere | 4 | 5 | |
| Tumor size (cm) | | | |
| ≤ 5 | 12 | 6 | 0.025 |
| > 5 | 3 | 9 | |
| Stage | | | |
| I + II A | 10 | 2 | 0.003 |
| II B/III | 5 | 13 | |
| Metastasis | | | |
| Yes | 4 | 12 | 0.003 |
| No | 11 | 3 | |

P value was acquired by Pearson chi-square test. The median expression level was used as the cutoff.

of *UCA1*, were transfected with pHLV Control/*UCA1* plasmid to overexpress *UCA1* (Fig. 1C) and MG-63 cells, which had the highest level of *UCA1*, were transfected with Control/*UCA1* shRNA to silence the *UCA1* expression (Fig. 1D). Results of CCK8 assays showed that *UCA1* overexpression significantly promoted cell proliferation (Fig. 1E) and there was a significant repression of growth in *UCA1* knockdown group (Fig. 1F) compared with the control groups respectively. Moreover, we found that overexpression of *UCA1* markedly inhibited the apoptosis, as shown by an increase of Annexin V-PE positive cells (Fig. 1G), whereas knockdown of *UCA1* increased the cell apoptosis (Fig. 1H). These results suggested that *UCA1* promotes the growth of osteosarcoma cells by increasing proliferation and decreasing apoptosis.

3.2. *UCA1* promotes the invasion and migration of osteosarcoma both *in vitro* and *in vivo*

To test the effect of *UCA1* on the invasion and migration of osteosarcoma cells, we introduced wound-healing and matrigel invasion assays as well as the Epithelial-Mesenchymal Transition (EMT) markers detection into U2-OS and MG-63 cells treated in the same way as above. We observed that when the endogenous *UCA1* was knocked down, MG-63 cells showed decreased capability of migrating to the monolayer of wounded cells (Fig. 2A) and invading through the matrigel treated transwell chambers (Fig. 2B). At the same time, the expression of epithelial marker E-cadherin was upregulated both in mRNA and protein levels, while the mesenchymal markers of Vimentin, Snail1 and ZEB1 were downregulated (Fig. 3A). On the contrary, when the ectopic *UCA1* was expressed, U2-OS cells presented increased migration and invasion ability compared with control cells (Fig. 2C and 2D) and the expression pattern of EMT markers were also opposite (Fig. 3A). To further explore the pro-metastasis effect of *UCA1 in vivo*, we established U2-OS cells that can stably express *UCA1* using lentiviral particles and injected these cells into the tail vein of nude mice. Four weeks later, we observed that compared to the control group, U2-OS cells overexpressing *UCA1* had stronger capability of invading and migrating to the lung, indicated by more (with the average number of metastasis nodules in *UCA1* overexpression group three times higher than that in control group) and larger metastatic nodules in the H&E staining of tissue sections observed under microscope (Fig. 3B). In summary, these data suggested that *UCA1* promotes the invasion and migration of

osteosarcoma cells both *in vitro* and *in vivo*.

3.3. *UCA1* directly interacts with miR-582 in osteosarcoma cells

Recent studies have indicated that lncRNAs exert regulatory functions through targeting miRNAs which bind to their 3'-untranslational regions (3'-UTR). After screening and comparison using online software program miRanda (<http://mircomrna.org>), we found that miR-582 had complementary base pairing with *UCA1* (Fig. 4A). We constructed reporter vectors (pmirGLO) containing the wild type (*UCA1*) and mutated 3'-UTR (*UCA1* Mut) of *UCA1* where miR-582 is binding. Results of the luciferase reporter assay showed that when we transfected 293T cells with miR-582 mimics together with *UCA1* vector, the luciferase activity significantly decreased whereas in the empty vector or *UCA1*-mut groups, no changes were observed (Fig. 4B). Next we confirmed the direct interaction between *UCA1* and miR-582 using RIP assay to pull down the endogenous miRNAs that interact with *UCA1* (Fig. 4C). The following qPCR analysis showed that compared to MS2 empty vector and *UCA1*-mut group, the *UCA1* RIP was significantly enriched for miR-582 in U2-OS cells (Fig. 4C). Since it is well defined that miRNAs bind to their targets and induce translational repression and/or RNA degradation through AGO2, a core component of the RNA-induced silencing complex (RISC), we performed anti-AGO2 RIP assay. Results showed high *UCA1* level in miR-582-overexpressing MG-63 cells when we use AGO2 to pull down the endogenous lncRNAs, suggesting the direct binding of miR-582 to *UCA1* (Fig. 4D). Furthermore, we found that ectopically expressed *UCA1* significantly reduced the miR-582 level (Fig. 4E) but overexpression of miR-582 did not alter the *UCA1* level (Fig. 4F), indicating that miR-582 could bind to *UCA1* but did not induce its degradation. Then we found the miR-582 level was significantly decreased, with the average expression 47.8% lower in tumor tissues than that in the corresponding normal tissues from 30 osteosarcoma patients (Fig. 4G), and the level of miR-582 was negatively correlated with the level of *UCA1* in the tumor tissues (Fig. 4H). All of the above data showed that *UCA1* directly interacts with *UCA1* in osteosarcoma cells.

3.4. *UCA1* functions as a ceRNA to upregulate CREB1 and promotes the metastasis of osteosarcoma cells through the AKT pathway

To further explore which gene related to the EMT of osteosarcoma cells is mediating the pro-metastatic effect of *UCA1* through interaction with miR-582, we scanned the possible target genes of miR-582 via the publicly available algorithms and focused on the gene CAMP Responsive Element Binding Protein 1 (CREB1). We analyzed the 3'-UTR of CREB1 which includes the putative binding site of miR-582 (WT 3'-UTR) and synthesized the mutated binding site (MT 3'-UTR) (Fig. 5A). After we sub-cloned these two 3'-UTR sequences into the reporter vector, we found the luciferase activity significantly decreased in miR-582 overexpressing MG-63 cells co-transfected with CREB1 WT vector and notably increased in U2-OS cells co-treated with miR-582 inhibitor and CREB1 WT vector compared to control groups (Fig. 5B) compared with the MT 3'-UTR groups, offering a direct evidence of miR-582 targeting CREB1. Then we found that overexpression of *UCA1* increased the level of CREB1 in both the protein and mRNA levels, while the co-transfection of miR-582 abrogated this upregulation (Fig. 5C). Meanwhile, we also observed increased proliferation, invasion and migration in *UCA1* overexpressing U2-OS cells (Figs. 5E, 6A and C). Similarly, the ectopic expression of miR-582 neutralized the enhancement of these malignant behaviors (Figs. 5E and 6A, C). On the contrary, we found the depletion of *UCA1* decreased the expression of CREB1 and the inhibition of miR-582 overcame this suppression (Fig. 5D). Also, the proliferation, invasion and migration of MG-63 cells were decreased with the downregulation of *UCA1* and restored with the following transfection of miR-582 inhibitor (Figs. 5F, 6B and D). All of the data suggested that *UCA1* functions as a ceRNA to upregulate

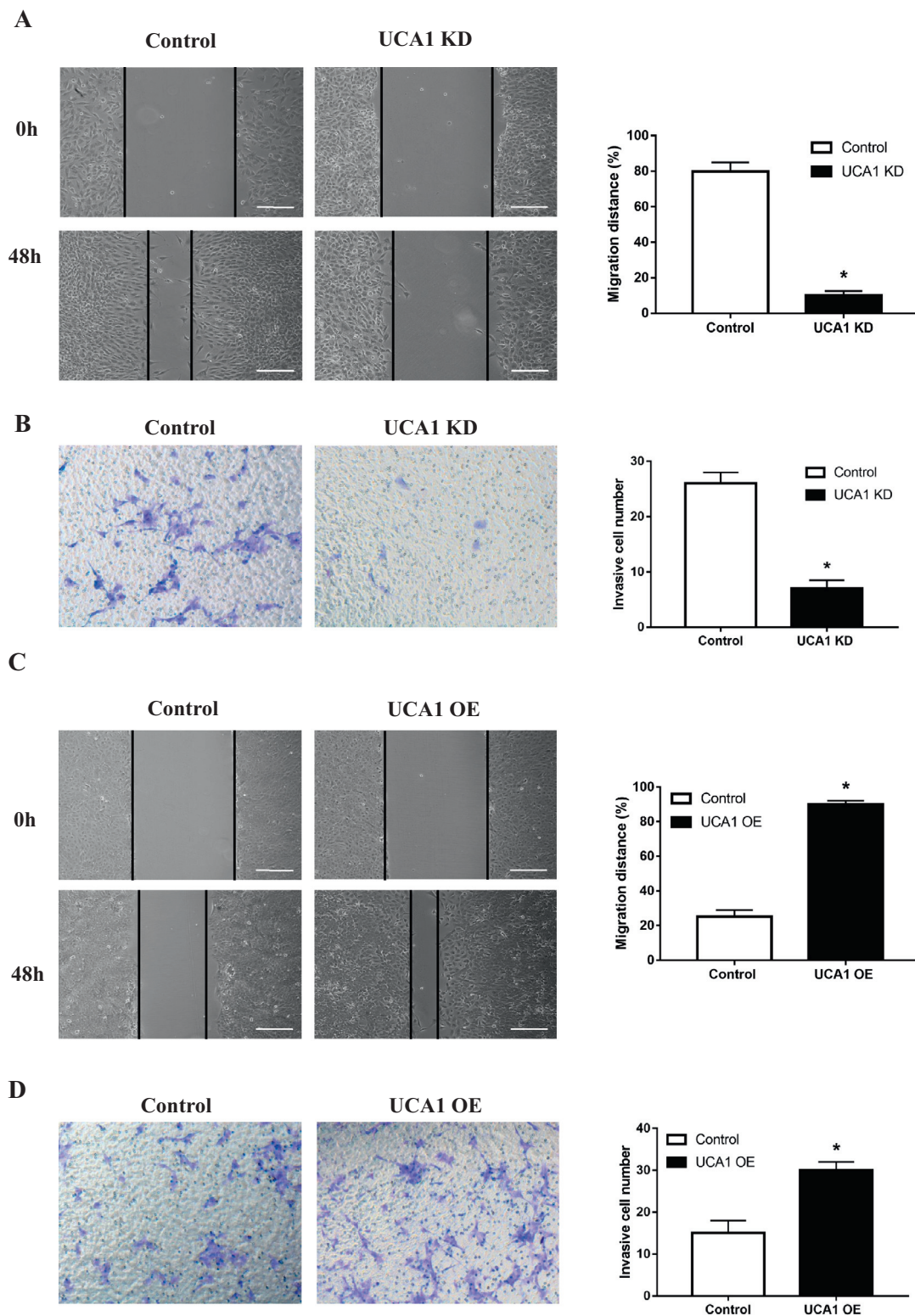


Fig. 2. *UCA1* promotes the invasion and migration of osteosarcoma *in vitro*. Wound-healing assays (A, C) and metrigel invasion assays (B, D) in MG-63 cells transfected with Control/*UCA1* shRNA (A, $p < 0.0001$; B, $p = 0.0002$) and U2-OS cells transfected with pHBLV Control/*UCA1* vectors (C, $p < 0.0001$; D, $p = 0.0002$). * $P < 0.05$ (Student's *t*-test).

CREB1 in osteosarcoma cells.

To further study how CREB1 mediates the pro-metastatic effect of its ceRNA *UCA1*, we tested the activity of AKT/mTOR pathway, which is closely related to the EMT and metastasis of osteosarcoma cells. Western blot analyses showed that when we overexpressed CREB1 in

U2-OS cells, both of the phosphorylated AKT and mTOR increased compared to the empty vector group, indicating the activation of this pathway (Fig. 6E, left). Meanwhile, the epithelial marker E-cadherin was downregulated and the mesenchymal marker Vimentin was upregulated (Fig. 6E, left). However, after we treated the CREB1-

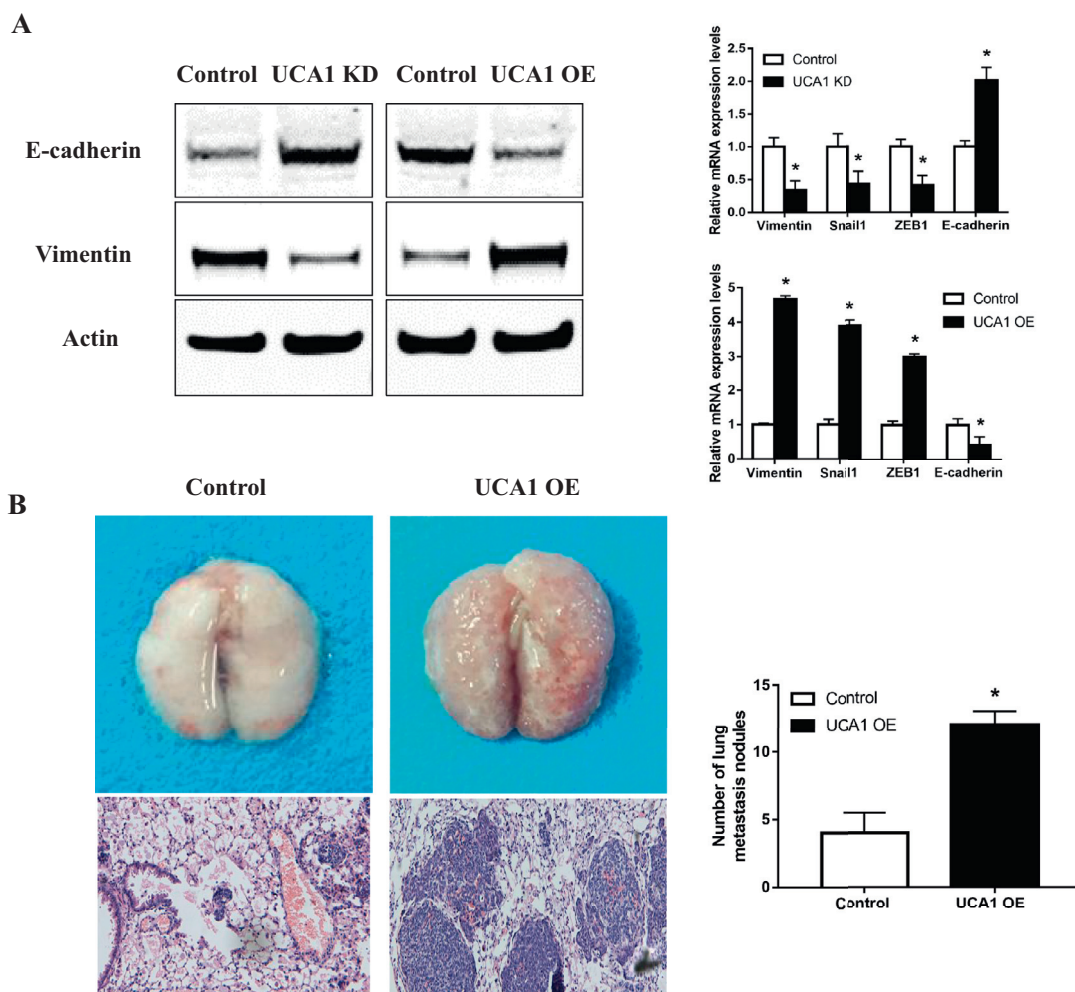


Fig. 3. *UCA1* promotes the invasion and migration of osteosarcoma *in vivo*. (A) Western blot and qPCR analyses of EMT markers in MG-63 cells transfected with Control/*UCA1* shRNA and U2-OS cells transfected with pHBLV Control/*UCA1* vectors (*UCA1* knockdown vs control: Vimentin $p = 0.0045$, Snail1 $p = 0.024$, ZEB1 $p = 0.0054$ and E-cadherin $p = 0.0013$; *UCA1* overexpression vs control: Vimentin $p < 0.0001$, Snail1 $p < 0.0001$, ZEB1 $p < 0.0001$ and E-cadherin $p = 0.0297$). (B) Lungs of nude mice in U2-OS/Lenti-Control and U2-OS/Lenti-*UCA1* groups 4 weeks after tail vein injection as labeled by H&E staining. The number of metastatic nodules per nude mouse was counted under a microscope ($p = 0.0015$). * $P < 0.05$ (Student's *t*-test).

overexpressing cells with DMSO(NC)/LY-294002, which is an inhibitor of the AKT/mTOR pathway, we found that besides the decreased phosphorylation of AKT and mTOR, the EMT process of U2-OS cells was also overturned (Fig. 6E, right). All of the above data suggested that CREB1 could promote the EMT and metastasis of osteosarcoma cells through activating the AKT/mTOR pathway, thus mediating the prometastatic effect of its ceRNA *UCA1*.

4. Discussion

LncRNAs can function as a vital regulator in a broad range of tumor biological processes, such as proliferation, apoptosis and migration. LncRNA *UCA1* is a recently identified noncoding RNA and dysregulation of *UCA1* is frequently observed in human cancers. Previous studies have shown that a high LncRNA expression is significantly correlated with poor osteosarcoma prognosis. The increased expressions of LncRNA XIST, SOX2-OT, MALAT1, HNF1A-AS1, PARTICLE, CCAL, 91H, HULC, BCAR4, TUG1, BANCR, MEG3 and HOTTIP indicate poorer prognoses of osteosarcoma patients, while the decreased expressions of LncRNA DANCR and TUSC7 indicate better prognoses of osteosarcoma patients. LncRNA *UCA1*, BCAR4, HULC, and MALAT1 are independent risk factors for poor overall survival rate in osteosarcoma patients. LncRNA DANCR, PARTICLE, HNF1A-AS1, XIST, CCAL and MEG3 can predict metastasis in osteosarcoma patients [11]. However, the role of *UCA1* in

regulating osteosarcoma invasion and migration remains unclear. In the present study, for the first time, we revealed that *UCA1* promotes the invasion and metastasis of osteosarcoma cells via suppression of miR-582 both *in vitro* and *in vivo*.

We firstly confirmed the increased expression of *UCA1* in osteosarcoma tissues and cell lines and clarified the positive correlation between *UCA1* level and the malignant features of clinical patients. The biological function tests showed that *UCA1* promoted the proliferation and inhibited the apoptosis of osteosarcoma cells. Importantly, we found that *UCA1* increased the invasive and metastatic capacities of osteosarcoma cells both *in vitro* and *in vivo*. Since tumor cells obtain migratory and invasive capabilities by EMT, which causes the cells to lose their intercellular adhesion [12] and to generate an aggressive tumor phenotype that leads to proximal or distant via direct invasion or lymphatic and circulatory systems [13], we detected the alterations of EMT markers following the increase or decrease of *UCA1* expression. Results showed that overexpression of *UCA1* significantly increased the expression of epithelial marker E-cadherin and decreased expression of the mesenchymal markers Vimentin, Snail and ZEB1, indicating the process of EMT was promoted. Conversely, knockdown of *UCA1* suppressed the process of EMT in osteosarcoma cells. For the first time, we demonstrated that *UCA1* promotes the process of EMT and thereby induces the invasion and metastasis of osteosarcoma cells.

It has been reported that lncRNAs exert their functions by

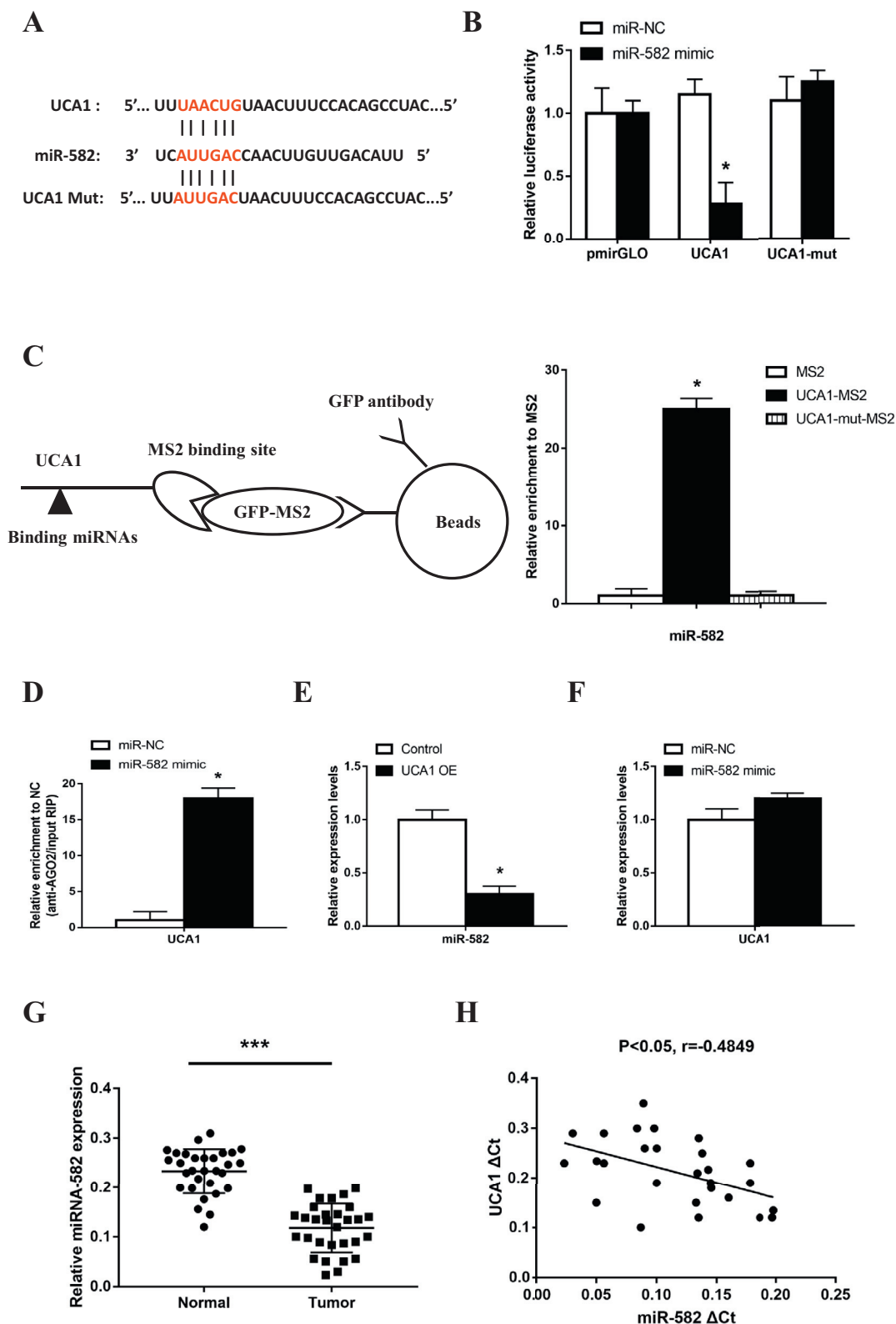


Fig. 4. *UCA1* directly interacts with miR-582 in osteosarcoma cells. (A) Diagram for the predicted binding site of miR-582 on the WT or Mut 3'-UTR of *UCA1*. (B) Luciferase reporter assays in 293T cells transfected with miR-NC/miR-582, together with pmirGLO empty vector or reporter vectors containing *UCA1*/*UCA1*-mut 3'-UTR (miR-582 vs miR-NC: pmirGLO $p > 0.99$, *UCA1* $p > 0.0019$, *UCA1* Mut $p > 0.284$). (C) Diagram and the qPCR analysis of the RIP assay performed in U2-OS cells to pull down the miRNAs associated with *UCA1* ($p < 0.0001$). (D) Anti-AGO2 RIP assay performed in MG-63 cells transfected with miR-NC/miR-582 followed by qPCR analysis ($p < 0.0001$). (E, F) Expression levels of miR-582 (E) and *UCA1* (F) detected by qPCR in *UCA1* overexpressing U2-OS cells (E, $p = 0.0005$) and MG-63 cells transfected with miR-NC/miR-582 (F, $p = 0.0663$). (G) Expression levels of miR-582 in paired normal and tumor tissues from 30 osteosarcoma patients detected by qRT-PCT ($p < 0.0001$). (H) The correlation between *UCA1* and miR-582 expressions in 30 osteosarcoma tissues ($p = 0.0104$). * $P < 0.05$ and *** $P < 0.001$ (Student's *t*-test).

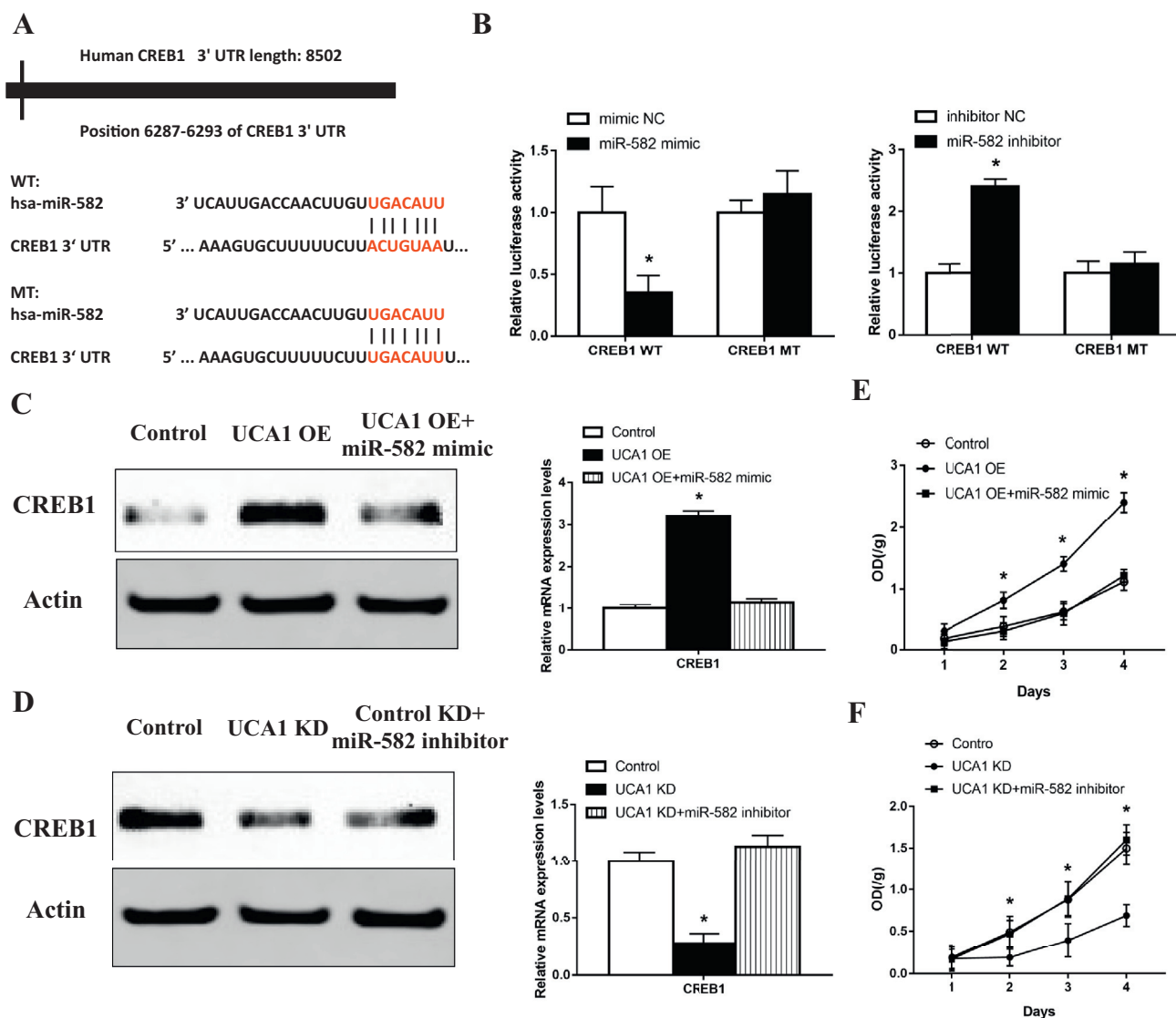


Fig. 5. *UCA1* functions as a ceRNA to upregulate CREB1. (A) Diagram of CREB1 3'-UTR containing the putative conserved target sequence for miR-582. (B) Results of luciferase reporter assays in MG-63 (left) and U2-OS cells (right) co-transfected with CREB1 WT/MT 3'UTR vectors and miR-NC/miR-145 (left) or inhibitor NC/miR-145 inhibitor (right) (miR-582 mimic vs mimic NC: CREB1 WT $p = 0.0112$, CREB1 MT $p = 0.2929$; miR-582 inhibitor vs inhibitor NC: CREB1 WT $p = 0.0002$, CREB1 MT $p = 0.3883$). (C-F) Expression levels of CREB1 (C, D) CCK8 assays (E, F) in U2-OS cells co-transfected with pHBLV control/pHBLV *UCA1* vectors and miR-NC/miR-582 (C, *UCA1* overexpression vs control $p < 0.0001$, *UCA1* overexpression + miR-582 mimic vs control $p = 0.1108$; E, day2: *UCA1* overexpression vs control $p = 0.0239$, *UCA1* overexpression + miR-582 mimic vs control $p = 0.548$; day3: *UCA1* overexpression vs control $p = 0.0016$, *UCA1* overexpression + miR-582 mimic vs control $p = 0.882$; day4: *UCA1* overexpression vs control $p = 0.0004$, *UCA1* overexpression + miR-582 mimic vs control $p = 0.371$) and in MG-63 cells co-transfected with Control shRNA/*UCA1* shRNA and inhibitor NC/miR-582 inhibitor (D, *UCA1* knockdown vs control $p = 0.0005$, *UCA1* knockdown + miR-582 inhibitor vs control $p = 0.1535$; F, day2: *UCA1* knockdown vs control $p = 0.0667$, *UCA1* knockdown + miR-582 inhibitor vs control $p = 0.896$; day3: *UCA1* knockdown vs control $p = 0.043$, *UCA1* knockdown + miR-582 inhibitor vs control $p = 0.955$; day4: *UCA1* knockdown vs control $p = 0.0038$, *UCA1* knockdown + miR-582 inhibitor vs control $p = 0.544$). * $P < 0.05$ (Student's *t*-test).

interacting with different molecules, such as miRNAs, mRNAs and proteins. Among them, the miRNAs can be targeted by binding to the 3'-UTR of lncRNAs and thus making the lncRNAs acting as a miRNA "sponge" or ceRNA [14]. Currently, the functional pattern of *UCA1* in osteosarcoma cells remains unclear. Recent studies have found that miR-582 acted as a tumor suppressor and could regulate bladder cancer progression both *in vitro* and *in vivo* [15]. However, the function of miR-582 in osteosarcoma still remains unclear. In the present study, for the first time, we demonstrated that *UCA1* acts as a ceRNA to sequester miR-582. Luciferase reporter and RIP assays demonstrated a direct interaction between *UCA1* and miR-582. We also found a negative correlation between *UCA1* and miR-582 expressions in osteosarcoma tissues. It is well known that miRNAs are key players in gene regulation

and perform functions relying on targeting genes. Using bioinformatic analysis, we found that the 3'-UTR of CREB1 mRNA had a complementary site for miR-582 binding. Luciferase reporter assay, western blot analysis and rescue experiments were performed to further confirm that CREB1 is indeed a target of miR-582 in osteosarcoma cells. Hence, we are the first to demonstrate that *UCA1* functions as a ceRNA of CREB1 through interacting with miR-582, thus suppressing its binding to the 3' UTR of CREB1 mRNA and increasing CREB1 expression in osteosarcoma cells. The PI3K/AKT/mTOR pathway is frequently activated and regulates cell survival, growth and migration in various tumors [16]. Although previous study has found that EMT could be mediated by PI3K/AKT/mTOR pathway [17], the effect of CREB1 on this pathway and the possible role of CREB1 in EMT still remain

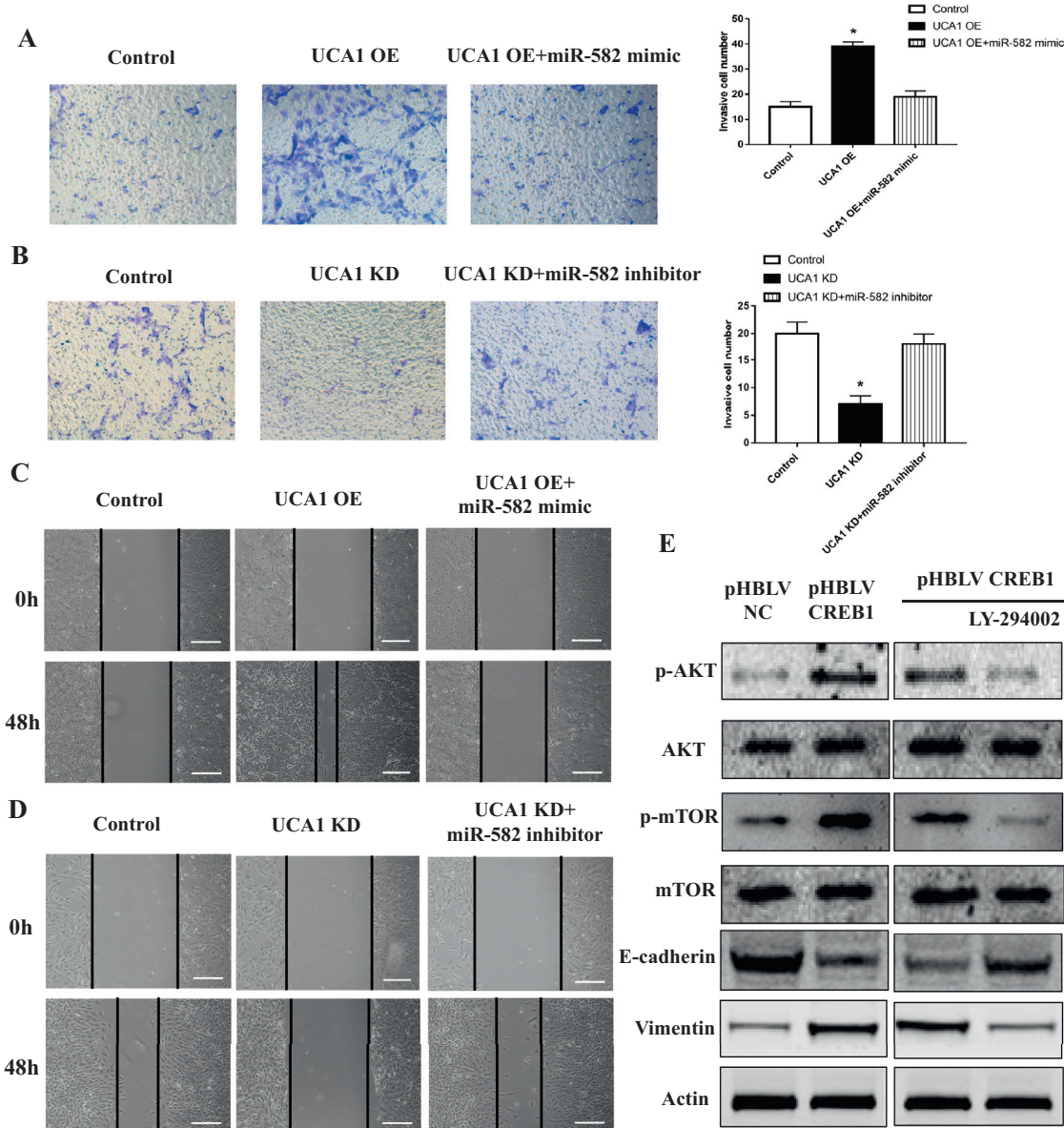


Fig. 6. *UCA1* functions as a ceRNA to upregulate CREB1 and promotes the metastasis of osteosarcoma cells through the AKT pathway. Metrigel invasion assays (A, B) and wound-healing assays (C, D) in U2-OS cells co-transfected with pHBLV control/pHBLV *UCA1* vectors and miR-NC/miR-582 (A, *UCA1* overexpression vs control $p = 0.0001$, *UCA1* overexpression + miR-582 mimic vs control $p = 0.09$; C) and in MG-63 cells co-transfected with Control shRNA/*UCA1* shRNA and inhibitor NC/miR-582 inhibitor (B, *UCA1* knockdown vs control $p = 0.0001$, *UCA1* knockdown + miR-582 inhibitor vs control $p = 0.288$; D). (E) Western blot analyses of total/p-AKT, total/p-mTOR, E-cadherin and Vimentin in U2-OS cells co-transfected with pHBLV control/pHBLV CREB1 vectors (left) and CREB1 overexpressing U2-OS cells treated with DMSO (NC)/LY-294002 (right). * $P < 0.05$ (Student's *t*-test).

unknown. For the first time, our results showed that CREB1 induced EMT of osteosarcoma cells by activating the PI3K/AKT/mTOR signaling pathway.

In conclusion, we confirmed that *UCA1* is upregulated in osteosarcoma and is related to more advanced clinical features. Importantly, our present study is the first to demonstrate that *UCA1* promotes the metastasis of osteosarcoma both *in vitro* and *in vivo*. Also, for the first time, we revealed that *UCA1*, as a ceRNA against miR-582, positively regulates the expression of its target CREB1, thus enhancing the EMT process via CREB1-mediated the PI3K/Akt/mTOR pathway and leading to the invasion and metastasis of osteosarcoma cells. These findings suggested that *UCA1* could be a potential biomarker and a promising therapeutic target for osteosarcoma treatment.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

GS designed the study. RS and HF performed the *in vitro* experiments. RS and YG performed the animal experimentation. HF and YG prepared the figures. RS, HF and YG collected and analyzed the data. GS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of No. 215 Hospital of Shannxi Nuclear Industry and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient consent for publication

The study was approved by the Medical Ethics Committee and written informed consents were obtained from all patients.

Competing interests

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2019.100228.

References

- [1] M.A. Smolle, M. Pichler, The role of long non-coding RNAs in Osteosarcoma, *Non-coding RNA* 4 (1) (2018) pii:E7.
- [2] D.R. Reed, M. Hayashi, L. Wagner, O. Binitie, D.A. Steppan, A.S. Brohl, E.T. Shinohara, J.A. Bridge, D.M. Loeb, S.C. Borinstein, M.S. Isakoff, Treatment pathway of bone sarcoma in children, adolescents, and young adults, *Cancer* 123 (2017) 2206–2218.
- [3] M. Huarte, The emerging role of lncRNAs in cancer, *Nat. Med.* 21 (2015) 1253–1261.
- [4] J. Huang, N. Zhou, K. Watabe, Z. Lu, F. Wu, M. Xu, Y.Y. Mo, Long non-coding RNA *UCA1* promotes breast tumor growth by suppression of p27 (Kip1), *Cell Death Dis.* 5 (2014) e1008.
- [5] Y. Han, Y.N. Yang, H.H. Yuan, T.T. Zhang, H. Sui, X.L. Wei, L. Liu, P. Huang, W.J. Zhang, Y.X. Bai, *UCA1*, a long non-coding RNA up-regulated in colorectal cancer influences cell proliferation, apoptosis and cell cycle distribution, *Pathology* 46 (2014) 396–401.
- [6] L. Zhang, X. Cao, L. Zhang, X. Zhang, H. Sheng, K. Tao, *UCA1* overexpression predicts clinical outcome of patients with ovarian cancer receiving adjuvant chemotherapy, *Cancer Chemother. Pharmacol.* 77 (2016) 629–634.
- [7] W. Nie, H.J. Ge, X.Q. Yang, X. Sun, H. Huang, X. Tao, W.S. Chen, B. Li, lncRNA-*UCA1* exerts oncogenic functions in non-small cell lung cancer by targeting miR-193a-3p, *Cancer Lett.* 371 (2016) 99–106.
- [8] X.Y. Na, Z.Y. Liu, P.P. Ren, R. Yu, X.S. Shang, Long non-coding RNA *UCA1* contributes to the progression of prostate cancer and regulates proliferation through KLF4-KRT6/13 signaling pathway, *Int. J. Clin. Exp. Med.* 8 (2015) 12609–12616.
- [9] X.S. Wang, Z. Zhang, H.C. Wang, J.L. Cai, Q.W. Xu, M.Q. Li, Y.C. Chen, X.P. Qian, T.J. Lu, L.Z. Yu, Y. Zhang, D.Q. Xin, Y.Q. Na, W.F. Chen, Rapid identification of *UCA1* as a very sensitive and specific unique marker for human bladder carcinoma, *Clin. Cancer Res. off. J. Am. Assoc. Cancer Res.* 12 (2006) 4851–4858.
- [10] Z.Q. Wang, Q. Cai, L. Hu, C.Y. He, J.F. Li, Z.W. Quan, B.Y. Liu, C. Li, Z.G. Zhu, Long noncoding RNA *UCA1* induced by SP1 promotes cell proliferation via recruiting EZH2 and activating AKT pathway in gastric cancer, *Cell Death Dis.* 8 (2017) e2839.
- [11] D. Chen, H. Wang, M. Zhang, S. Jiang, C. Zhou, B. Fang, P. Chen, Abnormally expressed long non-coding RNAs in prognosis of Osteosarcoma: a systematic review and meta-analysis, *J. Bone Oncol.* 13 (2018) 76–90.
- [12] H. Acloque, M.S. Adams, K. Fishwick, M. Bronner-Fraser, M.A. Nieto, Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease, *J. Clin. Investig.* 119 (2009) 1438–1449.
- [13] J.H. Hong, E.S. Hwang, M.T. McManus, A. Amsterdam, Y. Tian, R. Kalmukova, E. Mueller, T. Benjamin, B.M. Spiegelman, P.A. Sharp, N. Hopkins, M.B. Yaffe, TAZ, a transcriptional modulator of mesenchymal stem cell differentiation, *Science* 309 (2005) 1074–1078 (New York, N.Y.).
- [14] T. Xia, Q. Liao, X. Jiang, Y. Shao, B. Xiao, Y. Xi, J. Guo, Long noncoding RNA associated-competing endogenous RNAs in gastric cancer, *Sci. Rep.* 4 (2014) 6088.
- [15] K. Uchino, F. Takeshita, R.U. Takahashi, N. Kosaka, K. Fujiwara, H. Naruoka, S. Sonoke, J. Yano, H. Sasaki, S. Nozawa, M. Yoshiike, K. Kitajima, T. Chikaraishi, T. Ochiya, Therapeutic effects of microRNA-582-5p and -3p on the inhibition of bladder cancer progression, *Mol. Ther. J. Am. Soc. Gene Ther.* 21 (2013) 610–619.
- [16] T.A. Yap, M.D. Garrett, M.I. Walton, F. Raynaud, J.S. de Bono, P. Workman, Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises, *Curr. Opin. Pharmacol.* 8 (2008) 393–412.
- [17] D. Zhang, S. Wang, J. Chen, H. Liu, J. Lu, H. Jiang, A. Huang, Y. Chen, Fibulin-4 promotes osteosarcoma invasion and metastasis by inducing epithelial to mesenchymal transition via the PI3K/Akt/mTOR pathway, *Int. J. Oncol.* 50 (2017) 1513–1530.